THE SIZE OF INFLUENZA VIRUS*

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Shope (1) in 1931 first demonstrated that swine influenza virus could be passed through Berkefeld filters, and 3 years later Andrewes, Laidlaw, and Smith (2) found that the swine and W.S. strains of influenza virus would pass collodion membranes having an average pore size of 600 mμ and hence contained infectious particles probably less than 300 mμ in diameter. In more detailed ultrafiltration studies in 1936, Elford and coworkers (3) obtained a filtration end point of 160 mμ for the swine and W.S. strains and concluded that the probable size of the virus particles was 80 to 120 mμ. Confirmation of this size range was obtained the same year by Elford and Andrewes (4) in centrifugation studies which indicated a particle diameter for the W.S. virus of 87 to 99 mμ. A year later Hoyle and Fairbrother (5) showed that some 90 per cent of the activity of swine and human influenza mouse lung preparations was deposited on centrifugation at 13,000 R.P.M. for 1 hour.

The size of influenza virus was generally accepted as 80 to 120 mμ until 1941 when Chambers and Henle (6) and Bourdillon (7) reported the results of centrifuge and diffusion experiments which indicated that about 1 per cent of the virus in mouse lung preparations and a somewhat larger percentage in infectious allantoic fluid had a particle diameter of about 6 to 11 mμ. It was noted in the centrifugation experiments that frequently much more virus activity remained in the supernatant fluids than should have remained there if all of the virus were represented by particles 80 to 120 mμ in diameter; hence it appeared that at least a portion of the virus possessed a much smaller particle size. The view that a small amount of the virus in influenza virus preparations might have a particle diameter considerably smaller than that generally accepted appeared to receive support when, in experiments carried out in the writer's laboratory in collaboration with Chambers and Henle, it was found possible to demonstrate by means of the ultracentrifuge the actual existence in centrifugally purified preparations of the F 12 strain of influenza virus of amounts ranging up to about 10 per cent of a component having a sedimentation constant of approximately 30 S, and therefore a particle diameter of about 10 mμ (8). The existence of this material was further demonstrated by electron micrography.

Somewhat later, however, Friedewald and Pickels (9) showed that, in experiments in which influenza virus was sedimented in the presence and in the absence of a sucrose density gradient under otherwise comparable conditions, much less activity remained in the supernatant fluid when the sucrose density gradient was present. They esti-

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mated that, in the presence of sucrose, the virus activity sedimented at a rate comparable to that of a particle at least 60 mμ in diameter. The results indicated that the failure of influenza virus to sediment in the absence of a density gradient might be due to artificial mixing due to disturbances of convection. Since one of the main reasons for the assumption by Chambers, Henle, Lauffer, and Anderson (8) that virus activity was associated with the component having a particle diameter of about 10 mμ was the failure of some of the virus activity to sediment after the manner of particles 80 to 120 mμ in diameter, the results of the sedimentation of the virus in the presence of a sucrose density gradient appeared to throw some doubt upon the validity of this assumption. However, if sucrose should cause influenza virus to aggregate, the results of Friedewald and Pickels might be due to aggregation of virus rather than to the elimination of convection disturbances. This possibility appeared to be a very real one, since Friedewald and Pickels noted differences in the character of the sedimentation curves which suggested varying amounts of aggregation. A further possible complication was provided by the fact that Friedewald and Pickels worked with the PR8 and W.S. strains of influenza virus, whereas most of Chambers and associates' centrifugation experiments were conducted with the F 12 strain.

Recently, Taylor, Sharp, Beard, Beard, Dingle, and Feller (10) and Sharp, Taylor, McLean, Beard, Beard, Feller, and Dingle (11) reported that the PR8 and Lee strains of influenza virus possess particle diameters of about 80 and 100 mμ, respectively, and that no component having a particle diameter of about 10 mμ was demonstrable by means of the ultracentrifuge. Although these authors state that their conclusions are wholly dissimilar to those of Chambers and associates, it may be observed that the experimental centrifuge data obtained by the two groups of workers are nevertheless quite similar in certain respects. For example, the experiment of Taylor, Sharp, Beard, Beard, Dingle, and Feller (10), in which the rate of sedimentation of influenza virus chick red cell agglutinating activity was compared with the rate of sedimentation of the Shope papilloma virus, indicates that about 5 per cent of the influenza virus sedimented at a rate less than that of the papilloma virus. This result could be regarded as evidence that about 5 per cent of the influenza virus is smaller than the papilloma virus which has a particle diameter of about 40 mμ. A similar interpretation could be offered for the results obtained in the centrifugation of PR8 allantoic fluid for 1 hour at 27,000 *g*, following which about 25 per cent of the virus activity was found in the supernatant liquid. As a whole, the results of Taylor, Sharp, Beard, Beard, Dingle, and Feller are consistent with the wealth of earlier data which indicate that the major portion of the material in influenza virus preparations possesses particle diameters in the range of 80 to 120 mμ, but their data provide no additional information regarding the interesting problem as to whether a small amount of the virus exists in the form of material having a particle diameter of about 10 mμ. This problem assumes importance because of the possibility that the 10 mμ material might represent native virus which subsequently aggregates to form material having particle diameters of 80 to 120 mμ.

The differences in results and in interpretation of data presented by different investigators made necessary a further study on the size of influenza virus. In

addition, it appeared desirable to investigate the components present in different virus strains because of the ease with which a component having a sedimentation constant of about 30 S was demonstrated to exist in F 12 allantoic fluid (8) and the observation that no similar component could be found in PR8 virus preparations (10). The question as to whether preparations of virus having particle diameters of 80 to 120 mμ represent aggregates of smaller infectious units also required elucidation. In an effort to secure significant evidence regarding these questions, two general methods of approach were employed in the present work. Firstly, the sedimentation behavior of various preparations of the PR8 and F 12 strains of influenza virus in the presence and in the absence of a sucrose density gradient was studied. Secondly, the physical separation of the small and large components of infectious allantoic fluid was effected by means of differential centrifugation and the biological and physical properties of the two purified components were determined. The results obtained have made possible a rational explanation for the differences encountered in earlier work on the centrifugation of influenza virus.

Materials and Methods

Source of Virus.—The PR8 strain of influenza virus was generously supplied by Dr. T. Francis, Jr., of the University of Michigan. When received in the form of frozen and dried allantoic fluid, the strain had been isolated from ferret 198, passed 70 times in mice, 717 times in tissue culture, and 23 times in chick embryos. It was passed twice in chick embryos in this laboratory, and a large amount of allantoic fluid from the second passage was distributed in a large number of sealed ampules which were stored in a CO_2 ice box. The F 12 strain of influenza virus was kindly supplied in the form of infectious allantoic fluid by Dr. W. Henle of The Children's Hospital of Philadelphia. The virus was passed twice in chick embryos in this laboratory and a large amount of allantoic fluid from the second passage was distributed and stored as described for the PR8 strain. At weekly or bimonthly intervals, single ampules of the stock viruses were thawed and allowed to stand at room temperature for about 30 minutes with occasional stirring. A 10^{-5} dilution of the PR8 strain and a 10^{-4} dilution of the F 12 strain were prepared with sterile 0.1 M phosphate buffer at pH 7 and 0.1 or 0.2 cc. portions were injected through a small opening in the shell above the air sac into the allantoic sac of White Leghorn chick embryos brought to 10 days of age at 39°C. (12). The infected embryos were incubated at 36°C. for 36 to 48 hours, then chilled for 2 or more hours at 4°C., and the allantoic fluid was harvested and used as starting material (12).

Chicken Red Cells.—Red cells obtained from adult chickens by severing the cervical vessels were prepared as described by Hirst and Pickels (13). The cells were stored at 4°C. in a 15 to 20 per cent suspension in saline and were used within 1 week of preparation.

Red Cell Agglutination Titrations.—The titrations of chicken red cell agglutination (CCA) activity were carried out by Hirst's method (13) as modified for use in this

laboratory (14). Unfortunately, during the major portion of the present work a virus standard was not available for purposes of comparison, hence the values reported in this paper are not standardized.

Chick Embryo Titrations.—The titrations in chick embryos were carried out as described by Hirst (15), except that sterile 0.1 M phosphate buffer at pH 7 was used as a diluent in place of saline (16).

Mouse Titrations.—The titrations in mice were made as described by Lauffer and Miller (17), and weighted 50 per cent end points were calculated.

Nitrogen Determinations.—Since all of the nitrogen in solutions of materials sedimented two or more times at high speed consisted of protein nitrogen, total nitrogen determinations by the Nessler method were made on such solutions and the values obtained were regarded as protein nitrogen values (12). In all other cases the samples were treated with an equal volume of hot 10 per cent trichloroacetic acid, then quickly cooled and the precipitate collected by centrifugation. The precipitate was dissolved in a small volume of 0.2 N sodium hydroxide and precipitated with 5 per cent trichloroacetic acid. Analyses by the Nessler method were made on the washed precipitates. Because of the possibility that different preparations might contain different percentages of nitrogen, the concentrations of protein are usually expressed in terms of protein nitrogen.

High-Speed Centrifugation.—Two vacuum centrifuges of the air turbine type described by Bauer and Pickels were used (18). The centrifuge heads, which were 8 inches in diameter, each accommodated 14 Lusteroid tubes 3 inches in length and 0.75 inch in diameter at an angle of 33° from vertical. About 10 to 15 minutes were required for acceleration or deceleration, and centrifugation at 24,000 R.P.M. was continued for 20 or 30 minutes in some cases and for 2 hours in other cases.

Sedimentation Constants.—Determinations of sedimentation constants of the purified preparations were kindly made by Dr. M. A. Lauffer and Mr. H. K. Schachman by means of a Bauer-Pickels type ultracentrifuge (19, 20) equipped with a Svensson-Philpot (21, 22) optical system. The solutions used usually contained about 2 to 3 mg. of protein per cc. in 0.1 M phosphate buffer at pH 7. The constants corrected to water at 20°C. are given in Svedberg units. A Svedberg unit, symbolized by S, is a sedimentation rate of 10^{-13} cm. per second per unit centrifugal field.

Preparation of Sucrose Density Gradients.—To seven test tubes containing 0.5 cc. portions of the virus preparation were added 0.5 cc. portions of solutions containing gradually increasing amounts of sucrose. For example, in order to secure final solutions containing from 0 to 20 per cent sucrose there were added to the first tube 0.5 cc. of water and to the remaining six tubes 0.5 cc. portions of solutions containing 4, 8, 16, 24, 32, and 40 per cent, respectively, of sucrose. The contents of the seven tubes were then combined in a single 7.5 cc. Lusteroid centrifuge tube ½ × 2½ inches. The solution containing no sucrose was added first and the most dilute solution with respect to sucrose was next added slowly down the side of the tube and the remaining solutions were added in sequence in a similar manner. The mixing afforded as the more dense solutions flowed past the less dense solution served to provide an almost continuous sucrose density gradient. After standing for 30 to 60 minutes, the tube was placed in a metal adapter and centrifuged in the equipment described above. Exposure to sucrose solutions for the periods of time and at the concentrations used in the present work was not found to affect the virus activity appreciably.

EXPERIMENTAL

Sedimentation of Influenza Virus in Presence and Absence of a Sucrose Density Gradient

In a preliminary experiment, the relative rates of sedimentation of six different materials on centrifugation at 24,000 R.P.M. in the same angle centrifuge head for different periods of time were determined. The materials used were freshly harvested clarified PR8 allantoic fluid, freshly harvested clarified F 12 allantoic fluid, a centrifugally purified PR8 virus preparation at a concentration of 0.1 mg. per cc., centrifugally purified tobacco mosaic virus at a concentration of 5.2 mg. per cc., centrifugally purified bushy stunt virus at a concentration of 0.1 mg. per cc., and egg albumin at a concentration of 6.2 mg. per cc. The solutions and centrifuge head were maintained at a temperature of about 20°C. The time required for acceleration and deceleration

TABLE I

Relative Rates of Sedimentation of Different Materials under Comparable Conditions

	Starting material			To speed and down			15 min. at speed			30 min. at speed			60 min. at speed		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
PR8 allantoic fluid		205	$10^{-4.7}$		40	$10^{-3.0}$					2	$10^{-2.8}$		<2	$10^{-2.7}$
F 12 allantoic fluid		46	$10^{-6.0}$		20	$10^{-5.3}$		2	$10^{-3.7}$		<2	$10^{-2.5}$		<2	$10^{-3.1}$
Purified PR8 virus		237			49			2			<2			<2	
Tobacco mosaic virus	5.2			2.4			0.3			0.04			0.03		
Bushy stunt virus	0.10			0.09			0.04			0.02			0.01		
Egg albumin	6.2			5.9			6.1			5.9			5.7		

a, numbers represent milligrams of protein per cubic centimeter in upper 3 cc. of tube.
b, numbers represent CCA titer of upper 3 cc. of tube.
c, numbers represent 50 per cent weighted end points in mice of upper 3 cc. of tubes.

of the centrifuge was 11 minutes in each case. Following centrifugation for the designated periods of time, the upper 3 cc. of each solution were removed by means of a pipette. The rates of sedimentation were followed by infectivity and CCA activity determinations in the cases of the influenza viruses and by nitrogen determinations in the remaining cases.

It can be seen from the results presented in Table I that the virus activity in the PR8 and F 12 allantoic fluids and in the purified PR8 sample was removed approximately as rapidly as the tobacco mosaic virus was removed. Tobacco mosaic virus has a sedimentation constant of about 193 S (23). The bushy stunt virus, which has a sedimentation constant of 132 S and is approximately spherical with a diameter of 26 mμ (24), sedimented much less rapidly. Less than 10 per cent of the egg albumin was removed from the upper 3 cc. of the solutions.

Results similar to those described above were obtained in an experiment with clarified PR8 allantoic fluid and bushy stunt virus carried out in an analogous manner except with an acceleration time of 15 minutes and a centrifugation

speed of 15,000 R.P.M. For example, following the 30-minute period of centrifugation, the concentration of bushy stunt virus in the upper 3 cc. was reduced from 0.1 mg. per cc. to 0.07 mg. per cc., whereas in the case of the PR8 preparation the CCA activity was reduced from 274 to 6 units per cc. and the chick embryo 50 per cent infectivity end point was reduced from 10^{-9} to $10^{-6.5}$. However, in other experiments, in which the PR8 and F 12 strains of influenza virus in allantoic fluids and in purified preparations were sedimented for 30 minutes at 24,000 R.P.M., it was found that frequently there was only a small difference between the virus activity of the upper 3 cc. and that of the lower 1 cc. of the centrifuge tube. It seemed possible that in the experiments in which the virus sedimented rapidly it was present in an aggregated form, whereas when it failed to sediment rapidly the virus was present in an unaggregated form. An alternative explanation for the failure of the virus to sediment rapidly was provided by the possibility that disturbances due to convection might result in an almost uniform distribution of virus despite the centrifugal force. It appeared desirable therefore to study the sedimentation behavior of influenza virus under conditions of minimum convection, such as in the presence of a sucrose density gradient, for, if disturbances due to convection are not important, it should eventually be possible to carry out an experiment in which the presence or absence of a sucrose density gradient would have no effect on the rate of sedimentation of the virus.

Accordingly, a variety of influenza virus preparations were sedimented for 30 minutes at 24,000 R.P.M. in the absence and in the presence of sucrose density gradients containing maximum sucrose concentrations ranging between 10 and 25 per cent sucrose. The virus concentration in the upper 3 cc. was compared with that of the lower 1 cc. by means of infectivity tests in chick embryos. The results are presented in Table II. It can be seen that in every case in which a sucrose gradient was used there was a marked difference in the virus activity of the upper and lower portions of the solution and that in every case in which the same material was studied in the presence and absence of a sucrose gradient much more activity was removed from the upper portion of the solution when the sucrose gradient was present. In Experiments 1 to 4 an effort was made to eliminate aggregated material by centrifuging allantoic fluid for 30 minutes at 24,000 R.P.M. and using the upper 50 per cent of the solutions. The results indicate that little or no concentration of virus activity occurred in the absence of a sucrose density gradient, whereas a marked sedimentation of virus occurred in the presence of a sucrose density gradient. The use of allantoic fluids harvested only 36 to 40 hours after inoculation of the embryos had no appreciable effect on the results (Experiments 1, 2, and 4). It may be noted that the ultracentrifugally purified virus used in Experiment 6 was obtained from the same allantoic fluid that was used for the preparation of the supernatant fluid used in Experiment 3. In Experiment 7 the virus concentration was varied over a 10,000-fold range, but in each case a marked concentration of activity occurred in the presence of the sucrose gradient. This result indicates that over the range studied there was no progressive disaggregation or disso-

TABLE II

Effect of a Sucrose Density Gradient on the Sedimentation of Influenza Virus

Experiment No.	Virus preparation	Solvent	Negative logarithm of 50 per cent chick embryo titer of solution after 30 min. at 24,000 R.P.M.		Estimated difference in virus titer between top and bottom
			Top 3 cc.	Bottom 1 cc.	
1	Upper portion of 40-hour F 12 allantoic fluid after 30 min. at 24,000 R.P.M. Diluted 1:10	0.1 M phosphate buffer	5.7	5.8	—
1 *a*	" "	0.1 M phosphate + 0–25 per cent sucrose	4.1	5.8	50×
2	" "	" "	4.6	6.3	50×
2 *a*	Same as Experiment 1 except at a dilution of 1:10,000	" "	1.8	3.6	63×
3	Upper portion of 48-hour PR8 allantoic fluid after 30 min. at 24,000 R.P.M. Diluted 1:10	0.1 M phosphate buffer	5.8	5.5	—
3 *a*	" "	0.1 M phosphate buffer + 0–10 per cent sucrose	4.3	5.6	20×
4	Upper portion of 36-hour F 12 allantoic fluid after 30 min. at 24,000 R.P.M. Diluted 1:10	0.1 M phosphate buffer	5.6	5.7	—
4 *a*	" "	0.1 M phosphate + 0–10 per cent sucrose	4.5	5.8	20×
5	Centrifugally purified virus from 48-hour PR8 allantoic fluid. 10^{-6} gm. protein per cc.	0.1 M phosphate buffer	4.5	4.7	—
5 *a*	" "	0.1 M phosphate + 0–20 per cent sucrose	4.0	5.2	16×
6	" "	0.1 M phosphate	5.4	6.5	13×
6 *a*	" "	0.1 M phosphate + 0–10 per cent sucrose	3.6	5.5	80×
7	Centrifugally purified virus from 48-hour PR8 allantoic fluid but at 10^{-4} gm. protein per cc.	0.1 M phosphate + 0–25 per cent sucrose	3.5	5.5	100×
7 *a*	Centrifugally purified virus from 48-hour PR8 allantoic fluid but at 10^{-6} gm. protein per cc.	" "	3.6	6.1	320×
7 *b*	Centrifugally purified virus from 48-hour PR8 allantoic fluid but at 10^{-8} gm. protein per cc.	" "	0.0	2.5	320×

ciation of virus with the formation of much smaller active virus particles as the concentration of the virus was reduced.

During the course of preparing centrifugally purified samples of the PR8 and F 12 strains of influenza virus, in which the nature of the final products was

checked by sedimenation constant determinations by means of the ultracentrifuge, it was noted that, in addition to a major component having a sedimentation constant of about 600 S, a component having a sedimentation constant of about 30 S was regularly demonstrable in the F 12 virus preparations, whereas it was difficult or impossible to demonstrate the existence of this component in the PR8 virus preparations. The results indicated that the concentration of the 30 S component was considerably higher in the F 12 allantoic fluid than in the PR8 allantoic fluid, and hence that the former should be used in studies on the nature of the 30 S component. It appeared desirable to determine the sedimentation behavior in a sucrose density gradient of virus in a preparation known to contain an appreciable amount of the 30 S component.

Accordingly, 450 cc. of 40-hour F 12 allantoic fluid containing 46 CCA units per cc. were centrifuged for 2 hours at 24,000 R.P.M. The supernatant liquid was discarded and the pellets were dissolved in 4 cc. of 0.1 M phosphate buffer at pH 7. Following centrifugation at low speed for 5 minutes, the solution was found to contain 0.98 mg. of protein nitrogen and 3020 CCA units per cc. Examination in the ultracentrifuge indicated that about 10 per cent of the protein consisted of the 30 S component and about 90 per cent of the 600 S component. A portion of the solution was used to prepare a tube containing sucrose at a concentration varying from about 0 to 25 per cent. The tube was centrifuged for 105 minutes at 30,000 R.P.M., and then seven 1 cc. samples were removed beginning at the top of the tube. The seven samples were dialyzed against distilled water for 36 hours and then analyzed for nitrogen by the Nessler method and for virus activity by the CCA method. In addition, the 50 per cent infectivity end points in chick embryos of the top 1 cc. and the lower two 1 cc. samples were determined. It can be seen from the results given in Table III that each of the first six samples contained considerably less protein and CCA activity than existed in the bottom 1 cc. sample. The CCA activity and the chick embryo titer of the bottom 1 cc. were over 2000 and 5000 times, respectively, those of the upper 1 cc. Although the major portion of the 30 S component should have moved from the upper 1 cc., it should still be present in sample 6, yet the latter contained only 0.011 mg. more protein nitrogen per cc. and no additional virus activity as measured by the CCA test or by chick embryo titration. As a whole, the results indicate that virus activity is not associated with the 30 S component.

In another attempt to correlate virus activity with the 30 S component, an ultracentrifugally purified preparation of F 12 virus was studied in the sector centrifuge cell after the manner described by Lauffer (25) in work with tobacco mosaic virus. The virus preparation was obtained from 450 cc. of 48-hour F 12 allantoic fluid containing 97 CCA units per cc. by two successive centrifugations for 2 hours at 24,000 R.P.M. The final solution of 2.2 cc. contained 1.31 mg. of protein nitrogen and 11,460 CCA units per cc. The preparation was demonstrated to contain an appreciable amount (10 to 20 per cent) of the 30 S component by means of the ultracentrifuge. An 0.8 cc. portion of this solution was placed in the sector cell and centrifuged for 98 minutes' effective time at 33,000 R.P.M. The material in the upper compartment was carefully separated and the material in the lower compartment was washed out with two volumes

of 0.1 M phosphate buffer. A second 0.8 cc. portion of the original solution was placed in the sector cell and centrifuged for 17 minutes' effective time at 22,200 R.P.M. The materials in the upper and lower compartments were removed as described above and the four fractions from the two centrifuge runs were analyzed for nitrogen and CCA activity and titered in mice. It can be seen from the results presented in Table IV that in both cases the bulk of the protein and the virus activity were in the lower com-

TABLE III

Effect on the Protein N and Virus Activity of a Virus Preparation Containing a 30 S Component on Centrifugation in a Sucrose Density Gradient for 105 Minutes at 30,000 R.P.M.

No. of sample beginning at top following centrifugation	Protein N content	CCA activity	50 per cent chick embryo titer of solution
	mg. per cc.	*units per cc.*	
1	0.018	<1	$10^{-5.3}$
2	0.018	<1	
3	0.019	<1	
4	0.016	<1	
5	0.022	<1	
6	0.029	<1	$10^{-5.0}$
7	0.559	2256	$>10^{-9.0}$

TABLE IV

Sedimentation in the Sector Centrifuge Cell of a Purified Influenza Virus Preparation Containing a 30 S Component

Conditions of centrifugation	Fraction of sector cell	Protein N concentration	CCA activity	50 per cent mouse titer of solution
		mg. per cc.	*units per cc.*	
17 min. at 22,200 R.P.M.	Top	0.204	54	$10^{-5.3}$
	Bottom (diluted 1:3)	0.705	4660	$10^{-7.4}$
98 min. at 33,000 R.P.M.	Top	0.043	20	$10^{-4.8}$
	Bottom (diluted 1:3)	0.675	3200	$10^{-6.8}$

partment, although in the case of the centrifuge run for 17 minutes at 22,200 R.P.M. the protein concentration and virus activity in the upper compartment were somewhat higher than in the case of the centrifuge run for 98 minutes at 33,000 R.P.M. It may be noted that in both cases the specific virus activity of the protein in the lower compartment was over ten times that of the protein in the upper compartment. It can be calculated, assuming normal sedimentation behavior, that but little of the 30 S component should move out of the upper compartment on centrifugation for 17 minutes at 22,200 R.P.M. but that all or most of this component should move out on centrifugation for 98 minutes at 33,000 R.P.M. The fact that the material in the upper com-

partment possessed about the same specific virus activity in both cases can be regarded as an indication that virus activity is not associated with the 30 S component.

The Physical Separation and Specific Virus Activity of the 600 S and 30 S Components of F 12 Allantoic Fluid

The experiments involving the centrifugation of influenza virus in the presence of a sucrose density gradient and in a sector centrifuge cell indicate that the virus activity is associated with the 600 S component and not with the 30 S component. Unfortunately, the experiments were not wholly unambiguous, since, if virus activity were associated with the 30 S component and sucrose caused this component to aggregate, the virus would always appear to sediment as a heavy component in the presence of sucrose. In the case of the sector cell experiments, artificial mixing due to convection disturbances as well as varying degrees of virus aggregation appeared to exist as possible complicating factors. Fortunately, however, the experiments indicated that it might be possible to effect by differential centrifugation the separation of the 30 S component from the 600 S component, and hence to compare the specific virus activities of the two purified components. In addition, the preparation in quantity of the 30 S component in an essentially pure form would make it possible not only to compare the rate of sedimentation of protein with that of virus activity in the presence of a sucrose density gradient, but also to establish by means of sedimentation constant determinations in sucrose solutions whether or not the 30 S component becomes aggregated in the presence of sucrose. It appeared therefore of considerable importance to attempt to prepare essentially pure preparations of the 600 S and 30 S components of F 12 allantoic fluid.

Accordingly, one liter of 36-hour F 12 allantoic fluid was centrifuged for 2 hours at 24,000 R.P.M., and the pellets were dissolved in 94 cc. of 0.1 M phosphate buffer at pH 7. A small amount of insoluble material was removed by low-speed centrifugation for 5 minutes and the supernatant liquid was then centrifuged for 20 minutes at 21,000 R.P.M. The supernatant liquid was decanted from the large pellets and again centrifuged for 20 minutes at 21,000 R.P.M. The supernatant liquid was removed and centrifuged for 2 hours at 24,000 R.P.M. to yield six small pellets which were dissolved in 1.5 cc. of phosphate buffer. On examination in the ultracentrifuge, this solution was found to contain only a single component with a sedimentation constant of about 30 S. The preparation was also found to contain 0.304 mg. of protein nitrogen and less than two CCA units per cc. and to have a 50 per cent infectivity end point in chick embryos at $10^{-10.4}$ gm. of protein nitrogen. The large pellets obtained after 20 minutes' centrifugation at 21,000 R.P.M. were dissolved in 30 cc. of phosphate buffer and again centrifuged for 20 minutes at 21,000 R.P.M. to yield two large pellets. These were dissolved in 11.5 cc. of phosphate buffer and centrifuged at low speed to remove a small amount of insoluble matter. The preparation was found to contain 0.304 mg. of protein nitrogen per cc., 615 CCA units per cc., a 50 per cent chick embryo infectivity at $10^{-13.0}$ gm. of protein nitrogen, and to contain but a single component having

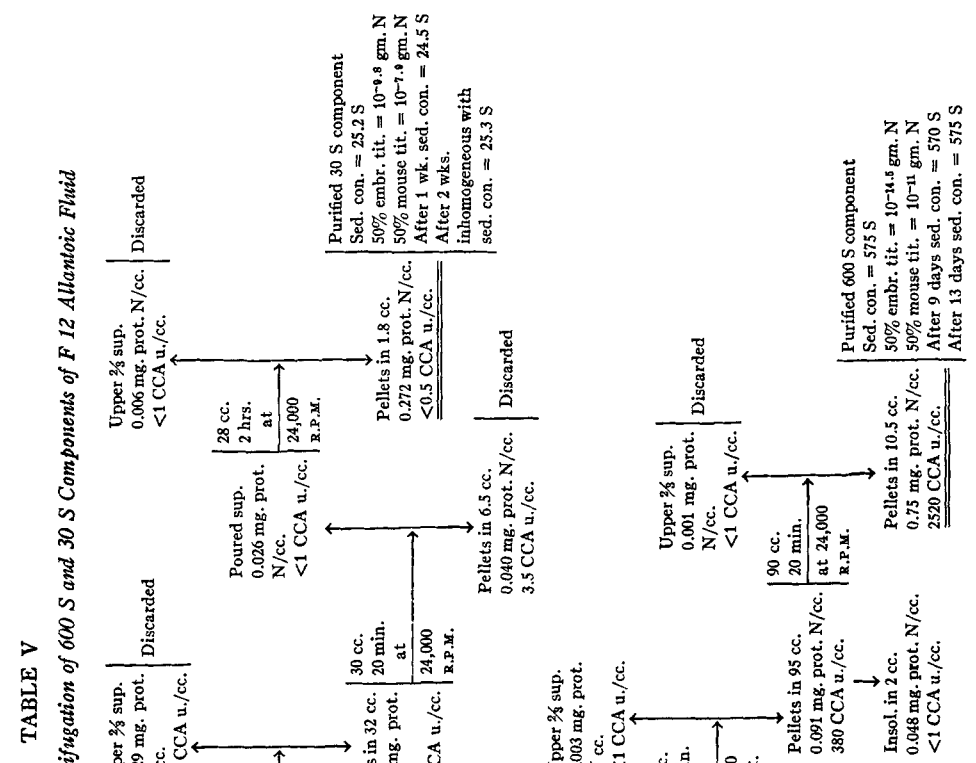
a sedimentation constant of about 600 S. In a repetition of the experiment with 1235 cc. of 40-hour F 12 allantoic fluid, the preparation of the small component was found to contain a total of 0.496 mg. of protein nitrogen, a sedimentation constant of about 30 S, no demonstrable CCA activity, and a 50 per cent chick embryo infectivity end point at $10^{-10.8}$ gm. of protein nitrogen. The corresponding preparation of the large component contained 4.0 mg. of protein nitrogen and possessed a sedimentation constant of about 600 S, 3180 CCA units per mg. of protein nitrogen, and a 50 per cent chick embryo infectivity end point at $10^{-13.7}$ gm. of protein nitrogen.

The two experiments demonstrate that it is possible to effect the physical separation of the 30 S and 600 S components by means of differential centrifugation. Only two fractionations proved sufficient to yield a 30 S preparation containing no 600 S material demonstrable in the ultracentrifuge and a 600 S preparation containing no demonstrable 30 S material. The ultracentrifuge results may be regarded as indicating that the 30 S preparation contained less than about 5 per cent of 600 S material and that the 600 S preparation contained less than about 5 per cent of 30 S material. If the virus activity of the 30 S preparation is regarded as being due to contamination with 600 S material, the fact that the specific virus activity of the 600 S preparation, whether measured by the CCA test or by means of chick embryos, was over 100 times that of the 30 S preparation, would indicate that the 30 S preparation contained less than 1 per cent of 600 S material. Although the results provide conclusive proof that two different orders of magnitude of specific virus activity are represented by the 600 S and 30 S preparations, they do not provide a sound basis for a decision as to whether the virus activity of the 30 S material is due to the presence of 600 S material as a contaminant or to 30 S material having a low specific virus activity. However, it appeared that this question could be answered by further fractionation of the 30 S preparation, for, if its activity should be due to contaminating 600 S material, it should be possible to decrease the proportion of this contaminant by fractionation in the centrifuge and thus to reduce further the specific virus activity of the 30 S preparation. For this purpose and in order to have sufficient of the final 30 S preparation for all tests, it was necessary to start with a larger amount of allantoic fluid than that used in the preceding experiments.

Accordingly, 2200 cc. of 40-hour F 12 allantoic fluid was fractionated during the course of 2 days, according to the diagram shown in Table V. The sedimentation constant determinations were made late on the 2nd day and all virus activity tests were made during the following morning. In addition, sedimentation constant determinations were made on both preparations after standing for different periods of time at 4°C. There was no change in the values and but little change in the homogeneity of the preparations at the end of 1 and 2 weeks, but thereafter both preparations became very inhomogeneous and in the case of the 30 S preparation it was impossible to demonstrate a sedimenting boundary. From the results shown in Table V, it may be

TABLE V

Separation and Purification by Differential Centrifugation of 600 S and 30 S Components of F 12 Allantoic Fluid



seen that the additional fractionation did further reduce the specific virus activity of the 30 S preparation, for it possessed less than 2 CCA units per mg. of protein nitrogen, a 50 per cent weighted end point in mice at $10^{-7.9}$ gm. of protein nitrogen, and a 50 per cent infectivity end point in chick embryos at $10^{-9.8}$ gm. of protein nitrogen, whereas the 600 S preparation possessed 3360 CCA units per mg. of protein nitrogen, a 50 per cent weighted end point in mice at $10^{-11.0}$ gm. of protein nitrogen, and a 50 per cent infectivity end point in chick embryos at $10^{-14.5}$ gm. of protein nitrogen. Thus, the CCA and mouse tests indicated that the virus activity of the 600 S preparation was over 1000 times that of the 30 S preparation and the chick embryo test indicated that the difference was over 10,000-fold. It is possible that the latter value more nearly represents the true difference in virus activity, since in the cases of the CCA and mouse tests it is necessary to use virus at a much higher level of concentration where the rôle played by inhibitory materials has not been fully evaluated (26).

The experiment described above provides a definite indication that the virus activity of the 30 S preparation is due to the presence of a very small amount (0.01 per cent or less) of the 600 S material, an amount which can be demonstrated only by virus activity tests and not by physical measurements. Additional evidence leading to the same conclusion was obtained in experiments in which the sedimentation constant of 30 S material was determined in the presence of sucrose and in which the sedimentation behavior in a sucrose density gradient of the virus activity of a 30 S preparation was studied. It was found by means of the ultracentrifuge that in solutions containing as much as 12 per cent sucrose the sedimentation constant of the 30 S material was not changed appreciably. This result provides conclusive proof that this material does not become aggregated in the presence of sucrose. In an additional experiment in which 30 S material at a concentration of 0.027 mg. of protein nitrogen per cc. was centrifuged for 20 minutes at 24,000 R.P.M. in the presence of a sucrose density gradient with a maximum sucrose concentration of 20 per cent, the virus activity, as measured in mice, of the lower 1 cc. was over ten times that of the upper 2 cc. Thus, the virus activity of the 30 S preparation sedimented as a heavy component under conditions under which the 30 S component is known to sediment normally and, hence, as a light component. As a whole, the results of the separation of the 600 S and 30 S components which are present in F 12 influenza virus allantoic fluid and the studies of the respective properties of the two purified components provide good reason for concluding that the virus activity is not associated with a component having a sedimentation constant of 30 S and a particle diameter of about 10 mμ, but is associated solely with the component having a sedimentation constant of about 600 S and a probable particle diameter of about 70 mμ.

DISCUSSION

In the present work it was found that, in accordance with the reports of earlier investigators, variable but significant amounts of the virus activity in

PR8 and F 12 allantoic fluids may or may not sediment as a heavy component on centrifugation in an angle centrifuge. In addition, the existence in F 12 allantoic fluids of appreciable amounts of a light component having a particle diameter of about 10 mμ has been confirmed, although this component was either not demonstrable or demonstrable with difficulty in PR8 allantoic fluids. However, the experiments described in the present paper render untenable the assumption that, in those cases in which the activity sedimented as a heavy component, the virus was present in an aggregated form, whereas, in those cases in which the activity failed to sediment readily, the virus was present in an unaggregated form, presumably the 10 mμ particles known to be present in F 12 allantoic fluid. It has been shown that, in the presence of a sucrose density gradient, influenza virus activity always sediments with a rate comparable to that of a heavy component having a particle diameter considerably greater than 10 mμ. This result indicates that the sucrose density gradient prevents or reduces artificial mixing of the contents of the centrifuge tube due to convection or mechanical disturbances and thus permits normal sedimentation. The possibility that sucrose causes the aggregation of influenza virus was rendered unlikely by the results of experiments in which it was shown that the small component having a sedimentation constant of 30 S and a particle diameter of about 10 mμ, which is present in F 12 allantoic fluid in an appreciable concentration, is not aggregated in the presence of sucrose. It was also demonstrated that the components present in F 12 allantoic fluid having sedimentation constants of about 600 S and 30 S could be separated by differential centrifugation and that the 600 S preparation possessed a specific virus activity from 100 to over 10,000 times greater than that of the 30 S preparation, the difference in activity apparently depending only on the degree of fractionation of the two components. This result indicates that the small residual virus activity present in the 30 S preparations was due to small residual amounts of the 600 S material present as a contaminant. The amount of 600 S material present as a contaminant was too small to be demonstrated by other than virus activity measurements. This interpretation was substantiated by an experiment in which the virus activity in a 30 S preparation was shown to sediment as a heavy component in the presence of a sucrose density gradient, whereas under the same conditions the 30 S component sedimented with its normal sedimentation rate. The results provide a rational explanation for the fact that influenza virus in dilute electrolyte solution frequently fails to sediment normally and render unnecessary the assumption that virus activity is associated with particles about 10 mμ in diameter. Furthermore, the results provide definite evidence that virus activity is not associated with particles about 10 mμ in diameter but is associated solely with material having a particle diameter of about 70 mμ.

The finding that a well defined component having a sedimentation constant of 30 S and a probable particle diameter of about 10 mμ is regularly present in

much higher concentration in the allantoic fluid of chick embryos inoculated with the F 12 strain of influenza virus, than in the allantoic fluid of embryos inoculated with the PR8 strain of influenza virus, is not without interest. It was estimated from ultracentrifuge diagrams that this component makes up about 30 per cent of the soluble protein material obtained on centrifugation of F 12 allantoic fluid for 2 hours at 24,000 R.P.M. In five separate experiments in which the 600 S and 30 S components were purified and separated from amounts of F 12 allantoic fluid ranging from 1000 cc. to 2550 cc., the 30 S component was found to represent from 7 to 12 per cent of the purified material. Since the losses involved in the purification by differential centrifugation of the 30 S component are considerably larger than those involved in the purification of the 600 S component, the actual yields of from 7 to 12 per cent would indicate that the proportion originally present in the F 12 allantoic fluids must have been at least that indicated by the ultracentrifuge diagrams or of the order of 30 per cent 30 S material to 70 per cent 600 S material. Although the presence of a few per cent of the 30 S component in purified PR8 virus preparations has been indicated occasionally by the ultracentrifuge diagrams, in many cases it has not been possible to demonstrate the existence of this component in PR8 virus preparations; hence its concentration in PR8 allantoic fluid must be much less than in the F 12 allantoic fluid. This finding provides an explanation for the fact that Chambers, Henle, Lauffer, and Anderson (8) were able to demonstrate the existence of a 30 S component in their purified influenza virus preparations, whereas Friedewald and Pickels (9) and Taylor, Sharp, Beard, Beard, Dingle, and Feller (10) were unable to do so, for the former worked with the F 12 strain whereas the latter worked with the PR8, W.S., and Lee strains of influenza virus. Although normal allantoic fluid contains characteristic protein components (27), the concentration of these appears to be less than that of the 30 S component in F 12 allantoic fluid. Hence most of the 30 S component appears to result from the influenzal infection and its occurrence in high concentration appears to be a special characteristic of the F 12 strain of influenza virus.

SUMMARY

The sedimentation behavior of influenza virus in dilute solutions of electrolyte was found to be quite variable. At times the virus activity appeared to sediment at a rate comparable with that of particles about 80 to 120 mμ in diameter, at other times at a rate comparable with that of particles about 10 mμ in diameter, and at still other times the bulk of the activity appeared to sediment at a rate comparable with that of the larger particles and the residual activity at a rate comparable with that of the smaller particles. However, in the presence of a sucrose density gradient, the virus activity was always found to sediment with a rate comparable to that of particles about 80 to 120 mμ in

diameter; hence it appeared that the variable sedimentation behavior in dilute electrolyte solution was due to convection or mechanical disturbances during centrifugation.

About 30 per cent of the high molecular weight protein present in the allantoic fluid of chick embryos infected with the F 12 strain of influenza virus was found to consist of a component having a sedimentation constant of about 30 S, and hence a probable particle diameter of about 10 mμ. The residual protein of high molecular weight was present in the form of a component having a sedimentation constant of about 600 S, and hence a probable particle diameter of about 70 mμ. The proportion of the 30 S component in allantoic fluid of chick embryos infected with the PR8 strain of influenza virus was found to be considerably less. The 600 S and 30 S components of F 12 allantoic fluid were purified and separated by differential centrifugation. The purified preparations of the 600 S component were found to possess a specific virus activity from 100 to over 10,000 times that of the purified preparations of the 30 S component, the difference in activity apparently depending only on the degree of fractionation of the two components. The purified 30 S component was found to sediment normally in the presence of 12 per cent sucrose, whereas the small residual virus activity of such preparations was found to sediment in the presence of a sucrose density gradient with a rate comparable to that of much heavier particles. It is concluded that influenza virus activity is not associated with material having a particle diameter of about 10 mμ, but is associated solely with material having a sedimentation constant of about 600 S and hence a probable particle diameter of about 70 mμ.

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